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Coexistence of Muscarinic Acetylcholine Receptors and Somatostatin in Nonpyramidal Neurons of the Rat Dorsal Hippocampus

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VAN DER ZEE, E. A., R. BENOIT, A. D. STROSBERG AND P. G. M. LUITEN. *Coexistence of muscarinic acetylcholine receptors and somatostatin in nonpyramidal neurons of the rat dorsal hippocampus*. BRAIN RES BULL 26(3) 343–351, 1991.—This study describes the colocalization of muscarinic acetylcholine receptors (mAChRs) and the neuropeptide somatostatin (SOM) in nonpyramidal neurons of the rat dorsal hippocampus. SOM and mAChRs were identified by immunocytochemistry employing antibody S309 and M35, respectively. Half of the SOMergic cell population is found to be immunoreactive for muscarinic receptor protein as obtained by fluorescent double-labeling techniques. These findings provide additional evidence for a direct cholinergic influence upon SOMergic, nonpyramidal neurons, and defines the anatomical distribution of SOMergic, cholinceptive neurons in the dorsal hippocampus. Concerning the muscarinic cholinceptive, nonpyramidal neuron population of the dorsal hippocampus, a considerable number (approximately one-third) was found to be colocalized with somatostatin. These results indicate that a significant part of the cholinergic influence upon hippocampal nonpyramidal neurons is relayed via SOMergic neurons.

Double-labeling immunocytochemistry
Dorsal hippocampus

Somatostatin

Muscarinic acetylcholine receptor

Rat brain

COMPLEX neuronal networks within the hippocampus are found to be related to learning and memory processes. There is ample evidence that the cholinergic septo-hippocampal projection, innervating all major cell groups of the hippocampus in a regional and laminar topography (13, 16, 17, 19, 24, 31, 40), is of significant importance in these functions (8, 23, 34). Muscarinic acetylcholine receptors (mAChRs) are considered to be the primary receptors in cholinergic synaptic transmission involved in learning and memory (7,10). Recently, muscarinic cholinceptive neurons have been visualized by immunocytochemical methods in our laboratory employing the monoclonal antibody M35 raised against purified mAChR proteins (50). From these methods, it became clear that muscarinic receptor immunocytochemistry provides an additional tool for further high resolution receptor study at the cellular and subcellular level.

Somatostatin (SOM) is one of the most important neuropeptides which interacts with the forebrain cholinergic system. Furthermore, SOM was found to be involved in a variety of behavioral functions including learning and memory (15, 19, 45). A subpopulation of hippocampal nonpyramidal neurons is somatostatinergic (SOMergic) in nature and their cellular distribution and morphology has been studied in detail (25, 27, 28, 33, 39, 52).

The interplay between the SOMergic and cholinergic system is characterized by modulatory effects of SOM on mAChR-functioning (35–37). However, the functional relevance of the interaction between the cholinergic and somatostatinergic system is still a matter of debate (22). Both systems show a consistent decline of activity in Alzheimer's disease (AD) (6, 14, 20, 44, 48). The reported decrease of activity of both transmitter systems in AD coincides with a significant to severe loss of hippocampal SOMergic neurons and breakdown of the cholinergic septo-hippocampal projection (12, 30, 43).

Presently, it is well established that pyramidal neurons and granule cells as well as some nonpyramidal neurons receive a cholinergic innervation from the medial septum-diagonal band complex, and therefore belong to the (muscarinic) cholinceptive system (13, 16, 17, 24). In addition, a subclass of the cholinergically innervated nonpyramidal neurons in the hilar region of the dentate gyrus was proven to be SOMergic in nature (27,29). In agreement with these observations, we recently demonstrated a monosynaptic septal input to SOMergic hippocampal neurons by *Phaseolus vulgaris* leuco-agglutinin (PHA-L) tracing (51). These tracing methods, however, do not permit a quantitative approach of SOMergic neurons receiving a cholinergic input. Also it re-

mains unclear which proportion of cholinceptive neurons is SOMergic in nature. In order to define the ratio of neurons being both SOMergic and muscarinic cholinceptive, we therefore studied the colocalization of somatostatin and mAChRs employing fluorescent immunocytochemical double-labeling techniques. In the present study we will demonstrate that about half of the population of SOMergic, nonpyramidal neurons in the dorsal hippocampus possess mAChRs. Consequently, these SOMergic neurons are under influence of cholinergic activity through mAChRs. This population of double-labeled cells comprises approximately one-third of the total number of muscarinic cholinceptive, nonpyramidal neurons in the dorsal hippocampus.

METHOD

Five young adult male Wistar rats (300 g body weight) were used in this study. Fixation of the brain was carried out by transcardial perfusion of 300 ml of fixative consisting of 3% paraformaldehyde, 0.05% glutaraldehyde and 0.2% picric acid in 0.1 M phosphate buffer (PB) at pH 7.4. Fixation was preceded by a prerinse with 50 ml saline solution at a perfusion speed of 25 ml/min, and followed by 100 ml of 10% sucrose in 0.1 M PB. The brains were removed, stored overnight in 30% buffered sucrose at 4°C for cryoprotection, and coronally sectioned on a cryostat microtome at a thickness of 20 microns.

Muscarinic acetylcholine receptors (mAChRs) and somatostatin were visualized by means of monoclonal antibody M35 raised against muscarinic receptor protein, and polyclonal antibody S309 directed against the first 14 amino acids of somatostatin-28, respectively. Extensive descriptions of production, characterization and immunocytochemical application of both antibodies have been previously reported (1–3, 5, 9, 38, 50).

Immunocytochemical Staining Procedure

From each rat, M35-single, S309-single, as well as S309/M35 double-labeling experiments were performed. For single labeling, the brain sections were incubated 24 h at 4°C in the primary antibody solution of phosphate buffered saline (PBS), containing mouse IgM anti-mAChR (M35, 1:2000) or rabbit IgG antisomatostatin (S309, 1:6000). Prior to both the primary and secondary antibody steps, the sections were preincubated in 10% normal rabbit serum or normal goat serum for M35 or S309, respectively. In the case of M35, the primary antibody step was followed by exposure to biotinylated rabbit anti-mouse IgM [Zymed, 1:200, 2 h at room temperature (RT)] and subsequently to streptavidin-HRP (Zymed, 1:200, 2 h at RT). For S309, the sections were incubated with goat anti-rabbit IgG (Zymed, 1:50, 2 h at RT), followed by rabbit-PAP (Dakopatts, 1:400, 2 h at RT). During all incubation steps of the S309 protocol, 0.5% Triton X-100 was added to enhance antibody penetration yielding an optimal labeling result. Finally, all tissue sections were processed by a diaminobenzidine (DAB)-H₂O₂ reaction (30 mg DAB and 0.01% H₂O₂/100 ml Tris buffer), guided by a visual check, and then mounted for light microscopic inspection.

Double-labeling experiments for the study of coexpression of mAChRs and somatostatin in single cells were carried out with fluorescent techniques. The sections were exposed to one of the primary antibodies as for single labeling described above. S309-incubation was followed by phycoerythrin-conjugated goat anti-rabbit IgG (Tago, 1:200, 2 h at RT). During all steps of the S309 protocol, 0.1% Triton X-100 was added to the incubation medium. This concentration of detergent was found to be the best compromise between no Triton X-100 (optimal condition for M35) and 0.5% Triton X-100 (optimal condition for S309). By reduc-

TABLE 1
NUMBER OF NEURONS COUNTED AND PERCENTAGE OF
COLOCALIZATION FOR M35 AND S309 IN THE DORSAL HIPPOCAMPUS
AS OBTAINED FROM FLUORESCENT LABELING

| Region | S309 ⁺ | S309 ⁺ /M35 ⁺ | (% Double Labeled) |
|-----------|-------------------|-------------------------------------|--------------------|
| Subiculum | 322 | 154 | (48) |
| CA1-CA3 | 1020 | 530 | (52) |
| Hilus | 736 | 362 | (49) |
| Total | 2078 | 1046 | (50) |

Single- and double-labeled SOMergic neurons in different areas of the dorsal hippocampus as obtained from fluorescent labeling. The percentage of SOMergic neurons colocalized with mAChRs reached about 50% in all hippocampal regions studied. M35⁺ = M35-immunopositive; S309⁺ = S309-immunopositive.

ing the amount of detergent to 0.1% as a compromise, the most weakly stained SOMergic hippocampal neurons may be overlooked in this study [unilaterally counted cells of a single section containing the dorsal hippocampus: 0.5% Triton X-100: 112 ± 1.2 (SE); 0.1% Triton X-100: 102 ± 1.5 (SE)]. Therefore, the proportion of coexpression found in this study may be slightly over- or underestimated. After completion of the S309 staining, the sections were incubated with M35, followed by biotinylated rabbit anti-mouse IgM and fluorescein isothiocyanate (FITC)-conjugated streptavidin (Zymed, 1:200, 2 h at RT).

After immunolabeling the sections were mounted and cover-slipped in a 1:1 mixture of PBS and glycerin. The sections were studied and photographed with a Ploemopak Leitz fluorescent microscope with the appropriate filter blocks for FITC and phycoerythrin labels, yielding a green and red fluorescence, respectively. In the case of a strong phycoerythrin signal, an additional faint, yellowish fluorescence was obtained under FITC filter which could easily be distinguished from the green FITC fluorescence.

Analysis

Standard control experiments were performed by omission of the primary antibody step, or by replacing the primary antibody by normal mouse serum or normal goat serum.

For quantification of the double-labeling experiments, we studied four to six sections per animal containing the hippocampus at the rostro-caudal level of bregma -2.8 to -3.8, according to the brain atlas of Paxinos and Watson (41). The hippocampus was divided in three regions studied on basis of the major localization of the SOMergic neurons: 1) subiculum, 2) stratum oriens of CA1-CA3 (Cornu Ammonis), and 3) the hilar region of the dentate gyrus. The quantitative data are presented in Table 1.

RESULTS

Muscarinic Cholinceptive Neurons

M35-immunolabeled hippocampal cells were observed to include pyramidal, granule and nonpyramidal neurons. Besides neurons, a minority of astrocytes was found to be M35 immunoreactive as well (Fig. 1C, 4C). Both cell bodies and dendritic arborizations were visualized by the M35 antibody (Fig. 1). Several classes of M35-positive nonpyramidal neurons were observed in the dorsal hippocampus. Strong immunopositive labeling appeared in a considerable number of pyramidal basket cells (long axis of the soma 10–20 µm; the size of these neurons averaged 16 µm) em-

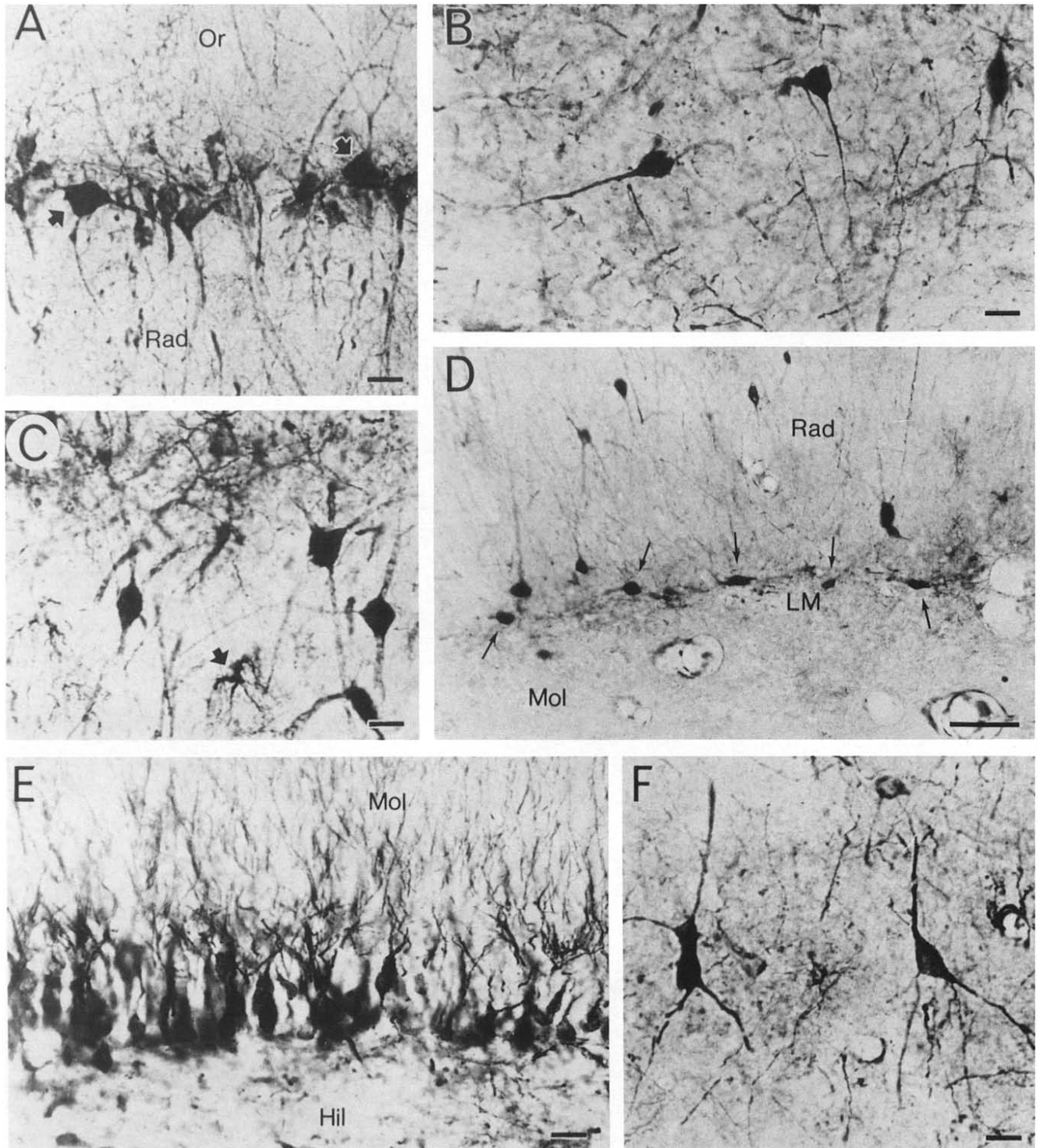


FIG. 1. Photomicrographs of M35-immunoreactive neurons in the dorsal hippocampus. (A) Nonpyramidal neurons (thick arrows) in the stratum pyramidale are larger and more intensely stained than the surrounding pyramidal cells. (B, C) Some typically nonpyramidal neurons and their dendritic processes in the subiculum (B) and stratum oriens (C). Thick arrow in (C): a M35-immunoreactive glial cell. (D) A sheet of M35-positive neurons in the stratum lacunosum-moleculare (thin arrows). (E) Granule cells and their extensive dendritic arborizations in the upper blade of the dentate gyrus. (F) Two examples of large neurons in the hilar region of the dentate gyrus. Scale bar in A,B,C,E,F = 15 μ m; in D = 50 μ m. Abbreviations here and in other figures: Gr: stratum granulare; Hil: Hilar region; LM: stratum lacunosum-moleculare; Mol: stratum moleculare; Or: stratum oriens; Pyr: stratum pyramidale; Rad: stratum radiatum.

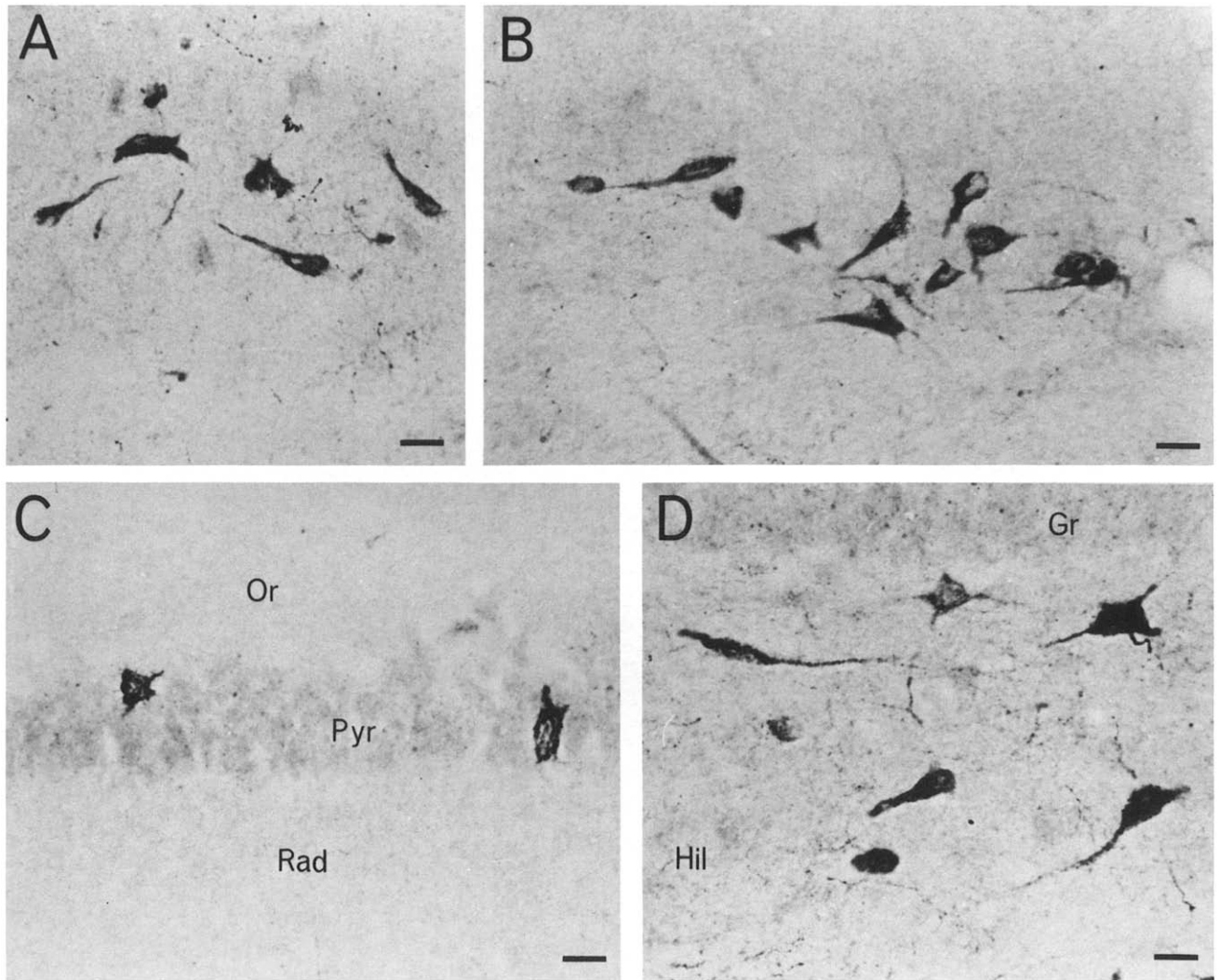


FIG. 2. Photomicrographs of S309-immunoreactive neurons in the dorsal hippocampus. (A) Cluster of relatively small neurons in the plexiform layer of the subiculum. (B) Clustered SOMergic, nonpyramidal neurons in the stratum oriens. (C) Some typical, but rarely observed SOMergic neurons embedded within the pyramidal cell layer. (D) Some large neurons in the deep hilar region of the dentate gyrus. Scale bar = 15 μ m. Abbreviations as in Fig. 1.

bedded within the CA1–CA3 pyramidal cell layer (Fig. 1A, 3A). Several forms of M35-positive nonpyramidal neurons were scattered throughout the subiculum (Fig. 1B), stratum oriens/alveus (Fig. 1C), and stratum lacunosum-moleculare (LM; Fig. 1D) of the Cornu Ammonis. In these regions, the neurons appeared to be medium to large sized (15–30 μ m). Most M35-positive neurons in the stratum LM had a somewhat spherical or spindle-shaped appearance, and were topographically arranged in a characteristic row (Fig. 1D, small arrows). These LM neurons were shown to be immunonegative for somatostatin.

In the dentate gyrus, a heterogeneous population of small (10 μ m) to large (30 μ m) muscarinic cholinceptive nonpyramidal neurons were observed (Fig. 1F). They were distributed throughout the polymorphic layer and the hilar region, as well as in the molecular layer. A multiform population of midsized to large (15–28 μ m) M35-positive basket cells was found either embedded in or adjacent to the hilar margin of the granule cell layer (Fig. 3C). Most of these neurons had pyramidal or somewhat

flattened cell bodies and were provided with horizontally oriented dendritic processes. Like the linearly arranged neurons in the stratum lacunosum-moleculare, the cells embedded in the hilar margin of the granule cell layer were immunoreactive for M35 only.

The distribution pattern of muscarinic cholinceptive nonpyramidal neurons proved to be very consistent in all animals studied. In contrast, the degree of M35-immunoreactivity in the main cell layers showed some individual as well as some regional variation.

SOMergic Neurons

The SOM-immunoreactive cell distribution visualized by the polyclonal antibody S309 revealed no differences as compared to previous findings (25,39), and all SOM-immunopositive neurons were of nonpyramidal class. In general the SOM-immunolabeled cells were distributed more in clusters (Fig. 2) than the scattered

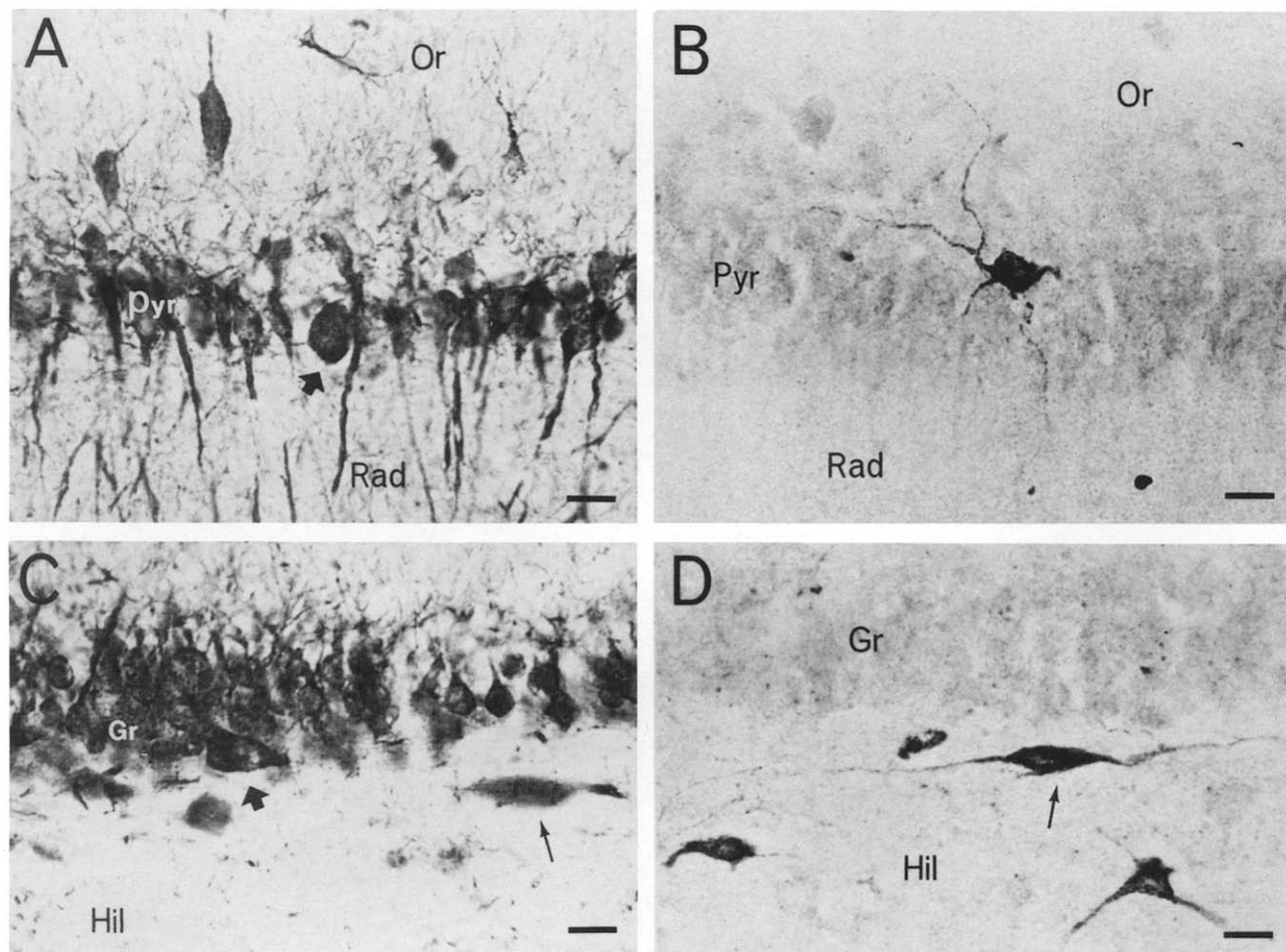


FIG. 3. Photomicrographs of some types of single-labeled M35- (left panel; A, C) and S309-immunoreactive neurons (right panel; B, D) to show direct comparison within the same hippocampal areas. (A–B) Basket-like nonpyramidal neurons situated within the pyramidal cell layer. The muscarinic cholinergic nonpyramidal neurons (thick arrow in A) are generally larger and more spherical in shape than their S309 counterparts. (C–D) M35-immunoreactive basket cells in the hilar region of the granule cell layer (thick arrow in C) were found to be S309-negative. Elongated M35- (C) and S309- (D) immunoreactive nonpyramidal neurons just beneath the granule cell layer, displaying striking resemblance in morphology (thin arrow). Scale bar = 15 μ m. Abbreviations as in Fig. 1.

M35-immunoreactive nonpyramidal neurons. In the dorsal hippocampus, SOM-immunopositive multipolar and spindle-shaped neurons, including medium-sized (15–20 μ m) and large cells (25–30 μ m), were predominantly found in the plexiform layer of the subiculum, the CA1–CA3 stratum oriens/alveus and the polymorphic and deep hilar region of the dentate gyrus. Some SOMergic neurons in the stratum oriens showed a clear difference in morphology as compared to the M35-positive neurons. For example, a part of these SOMergic neurons appeared to be bipolar, spherical cells with thick proximal dendritic processes (Fig. 4D). This class of neurons appeared to be devoid of muscarinic acetylcholine receptors. As is the case with M35-positive nonpyramidal neurons, some SOMergic cells were found to be localized within the CA1–CA3 pyramidal cell layer (Fig. 2C, 3A, B). However, they differed in morphology, were less numerous and smaller than the M35-immunoreactive nonpyramidal neurons, ranging from 7.5 to 15 μ m in size (average 10 μ m). Only rarely the largest neurons of this population appeared to be muscarinic cholinergic. As mentioned above, no SOMergic neurons were found in the

stratum lacunosum-moleculare.

SOMergic, Muscarinic Cholinergic Neurons

The partial resemblance in morphology and distribution of the two cell populations already indicated a tentative coexpression of mAChRs and SOM in some nonpyramidal neurons in the three hippocampal areas studied. Such a striking resemblance is illustrated in Fig. 3C–D showing a similarly looking M35- and S309-immunoreactive nonpyramidal neuron in the hilar region of the dentate gyrus (small arrow). Fluorescent double-labeling experiments affirmed the putative colocalization of these two substances. This class of hilar nonpyramidal neurons shown in Fig. 3C–D just beneath the granule cell layer indeed coexpressed mAChRs and somatostatin (Fig. 4E–F). Such double-labeled neurons were found in all dorsal hippocampal regions containing SOMergic cells. Double-labeled as well as single-labeled (single SOMergic or single muscarinic cholinergic) nonpyramidal neurons were distributed amongst each other in the plexiform layer of the subiculum,

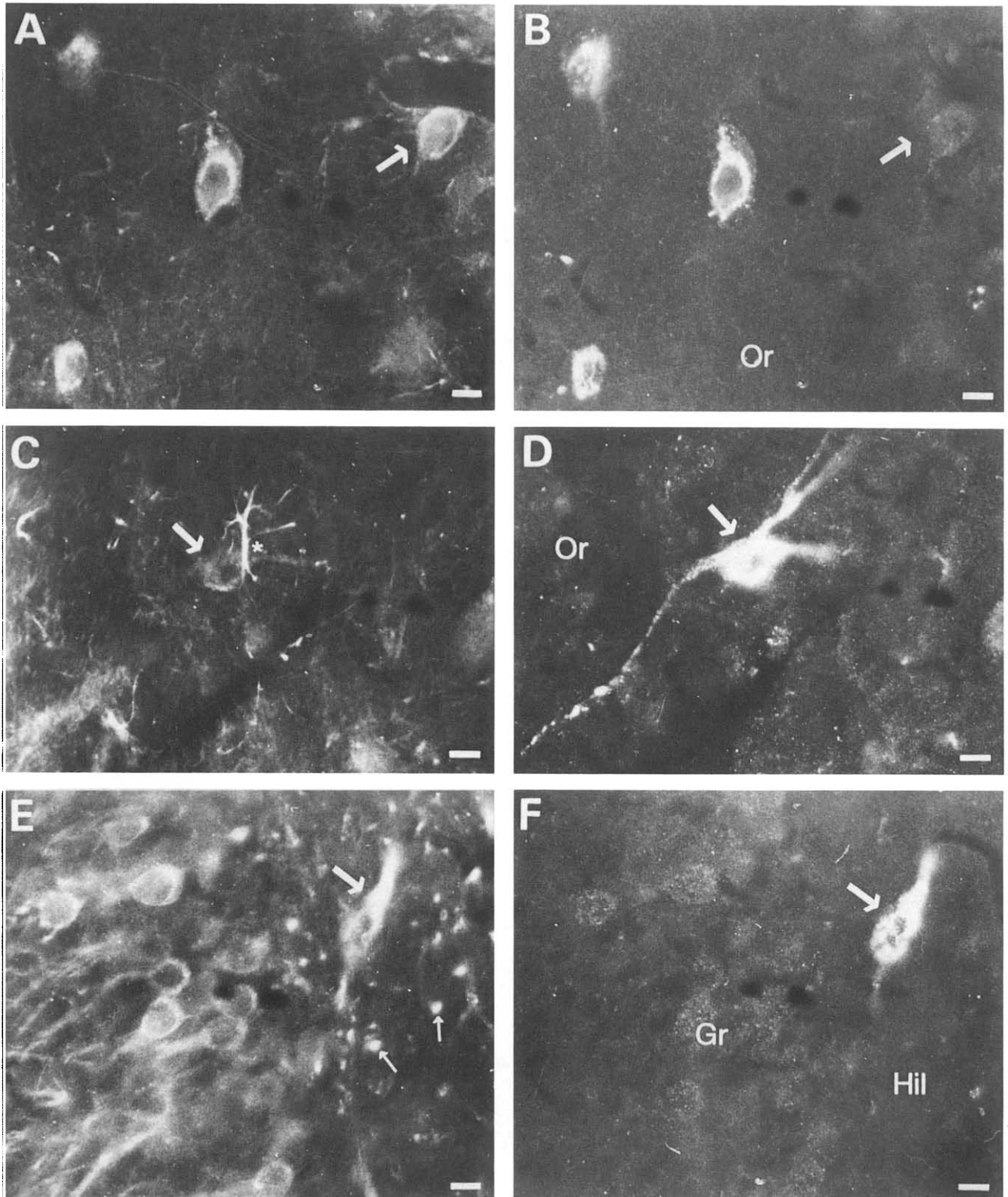


FIG. 4. Fluorescent photomicrographs depicting double-labeled neurons in the dorsal hippocampus. (A, C, E) Neurons labeled with FITC for M35; (B, D, F) Neurons labeled with phycoerythrin for S309. The arrows in A to D indicate single labeled nonpyramidal neurons in the stratum oriens (A-B: M35, C-D: S309). Adjacent to the SOMergic cell in D, a M35-immunoreactive glial cell in C (asterisk) is present, which is clearly devoid of S309 immunoreactivity (D). Under FITC filter, the strong phycoerythrin-labeled SOMergic cell (D) shows a yellowish fluorescence (arrow in C). In E-F a characteristic hilar nonpyramidal neuron (arrow) is double labeled. This neuron strongly resembles the one shown in Fig. 3C-D. The small arrows point at transsectioned dendritic processes, which are M35-immunopositive only. Scale bar = 10 μ m. Abbreviations as in Fig. 1.

the CA1–CA3 stratum oriens (Fig. 4A–B, C–D) and the polymorphic and deep hilar region of the dentate gyrus (Fig. 4E–F). Moreover, no consistent, discernible morphological differences between the cholinceptive and noncholinceptive SOMergic neuronal subpopulations distinguished in this study were found.

Per single 20 μ m section of the dorsal hippocampus, unilaterally cell counts of SOMergic neurons revealed an average of 102 ± 1.5 (SE) neurons (numbers ranging from 90–110). These cell counts showed that half of this SOMergic cell population possesses muscarinic acetylcholine receptors. This proportion slightly fluctuated in a random fashion for adjacent sections and between left and right dorsal hippocampi. Nearly identical proportions of colocalization were found in the different hippocampal regions investigated. Furthermore, data pooled per animal revealed a consistent coexpression of about 50% in all animals. Therefore, average numbers were calculated from collected data obtained from all dorsal hippocampi studied (Table 1).

Cell counts per 20 μ m sections obtained from DAB-processed hippocampal sections revealed an average of $114 [\pm 1.3$ (SE)] and $153 [\pm 1.1$ (SE)] neurons immunopositive for S309 and M35, respectively. Since half of the SOMergic neurons are muscarinic cholinceptive, they in turn made up about one-third of the total number of M35-immunoreactive nonpyramidal neurons in the hippocampus at the rostral-caudal level studied.

DISCUSSION

The results presented here demonstrated half of the SOMergic hippocampal cell population to be immunoreactive for muscarinic receptor protein. As such, these findings provide additional evidence for a direct cholinergic influence upon SOMergic, nonpyramidal neurons, and define the anatomical distribution of SOMergic, cholinceptive neurons in the dorsal hippocampus. Concerning the muscarinic cholinceptive, nonpyramidal neuron population of the dorsal hippocampus, a considerable number (approximately one-third) was found to be colocalized with somatostatin. These results indicate that a significant part of the cholinergic influence upon hippocampal nonpyramidal neurons is relayed via SOMergic neurons.

An interaction between the cholinergic and somatostatinergic systems has also been reported for the cerebral cortex and the striatum. Somatostatin was released in an atropine-sensitive manner after acetylcholine application to cultured cerebral cortical neurons, suggesting involvement of mAChRs (46). Striatal muscarinic acetylcholine receptors were also found to be associated with SOMergic neurons (4). In agreement with these studies, we found SOMergic neurons both in the cerebral cortex and striatum to be colocalized with mAChRs in our double-labeling experiments (data not shown).

A functional implication of the cholinergic influence upon SOMergic hippocampal neurons may lie in feed-forward and feed-back inhibitory processes. The SOMergic neurons are considered to be a subpopulation of inhibitory gamma-aminobutyric

acid (GABA)ergic cells. Some studies claim that as much as 90% of the SOMergic hilar neurons is immunoreactive for GABA (26,49). It is concluded that these SOMergic cells serve both feed-forward and feed-back inhibitory processes in the dentate gyrus (11,29). Furthermore, cholinergic afferents to GABAergic hilar basket cells are implicated in feed-forward inhibition (28,29). Taken together, the SOMergic muscarinic cholinceptive neurons found in this study are likely candidates for inhibitory processes driven by the excitatory cholinergic septal input.

Besides a cholinergic influence upon SOMergic neurons through mAChRs as discussed above, a more direct interplay between somatostatin and mAChRs is reported by Miyoshi and co-workers (35–37). In tissue homogenates, somatostatin, acting through its own receptors, reduces the affinity of the agonist binding of the M1 mAChR-subtype in the hippocampus, thereby affecting the synaptic transmission of acetylcholine. In this way, an inhibitory modulatory effect on cholinergic hippocampal activity is suggested.

Finally, the (muscarinic) cholinceptive principal and SOMergic interneurons are simultaneously innervated by the septal cholinergic neurons (13, 16, 17, 24). The functional implication of a combined release of somatostatin and acetylcholine upon target cells will vary, depending on the quantity and temporal context (32).

Research on neurotransmitter deficits in Alzheimer's disease (AD) showed a consistent decrease in cholinergic and somatostatinergic activity (6, 12, 14, 20, 44, 48). However, the precise mechanisms of interaction between both systems remain unclear. SOMergic immunoreactivity is not influenced by cholinergic denervation of the rat hippocampus (42), and long-term cholinergic denervation of the cortex was even shown to induce a significant increase of SOM immunoreactivity (18). Nevertheless, it is tentative to speculate on a crucial role of the SOMergic muscarinic cholinceptive neurons as a morphological substrate involved in learning and memory function of the hippocampus. Future research may further elucidate possible differences in vulnerability of the two SOMergic cell populations as distinguished in this study, in AD.

In conclusion, half of all dorsal SOMergic hippocampal neurons belong to the muscarinic cholinceptive cell population. The SOMergic muscarinic cholinceptive cell group is randomly distributed between the entire SOMergic cell population throughout the dorsal hippocampus, and includes approximately one-third of the total muscarinic cholinceptive nonpyramidal neurons. No characteristic laminar or regional organization was observed in relation to its SOMergic nonmuscarinic cholinceptive counterpart. Future research may elucidate the possibility of the two different SOMergic subpopulations found in this study to be functionally distinct groups of neurons.

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